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小鼠甲胎蛋白单克隆抗体的制备及其初步应用研究

Preparation of monoclonal antibodies against mouse alpha-fetoprotein and preliminary study on their application

潘海娇

指导教师姓名：罗文新 副教授

专 业 名 称：水生生物学

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潘海娇
指导教师 罗文新 副教授
厦门大学

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摘 要

原发性肝癌是我国高发的恶性肿瘤之一，每年新发和死亡患者均占全球总数的一半以上。根据全国肿瘤登记中心最新公布的 2015 年《肿瘤登记年报》数据，我国肝癌新增病例为 46.61 万，占所有恶性肿瘤的第 4 位（10.86%），而死亡病例为 42.21 万，占所有癌症死亡的第 3 位（15.00%），仅次于肺癌和胃癌。尽管近年来肝癌的诊断和治疗有了一定的进展，其预后仍然不理想，五年生存率极低。究其原因，大部分肝癌患者往往在疾病的晚期才被诊断出来，有些甚至发生了远端转移，因而错过了最佳治疗时机；此外，目前几乎没有针对中晚期肝癌有效的治疗手段。在这种情况下，迫切需要开发肝癌早期诊断试剂及新型靶向药物，提高治疗效率。

甲胎蛋白（alpha-fetoprotein, AFP）是一种分泌型的糖蛋白，通常特异性地在胚胎发育过程中的肝细胞或肝癌细胞内高表达，而在健康成体的肝细胞内不表达。AFP 是目前临床上应用最广的原发性肝癌肿瘤标记物，血清中 AFP 水平常用于肝癌的普查、早期诊断、术后监测和随访。尽管 AFP 对肝癌病人的诊断灵敏度仅约 60%，但它仍然是目前为止最为理想的原发性肝癌诊断的临床指标。目前研究者们正在致力于开发新的原发性肝癌辅助诊断标记物，以期联合 AFP 提高原发性肝癌的诊断灵敏度。此外，针对 AFP 蛋白的表位疫苗也在化学诱导的肝癌模型中被证实能诱导机体产生针对肝癌细胞内 AFP 的 $CD8^{+}T$ 细胞免疫应答。AFP 抗原也被逐渐认为是肝癌免疫治疗的潜在治疗靶标。

原发性肝癌动物模型是评估肝癌靶向药物临床转化价值的理想模型。原发性肝癌模型的特点是其发生过程类似于人肝癌发生的恶性转化过程，具备正常的免疫系统，在研究肝癌进程的不同阶段的病理变化特点和疗效评估具有独特的优势。目前原发性肝癌小鼠模型建模周期长（8 个月以上），迫切需要发展短期建模的小鼠肝癌模型。更重要的是，目前急缺原发性鼠肝癌筛查、诊断及疗效评估的有效检测方法，鼠 AFP 作为小鼠肝癌的最特异的肿瘤标记物，针对小鼠 AFP 建立检测方法具有重要的研究价值。

本研究旨在获得具有诊断价值的抗小鼠 AFP 单克隆抗体，并在原发性小鼠肝癌模型中初步探索 AFP 单克隆抗体应用于肝癌检测、诊断和治疗的可行性。我们利用 Gibson 装配技术获得 mAFP 重组质粒后，体外重组表达和纯化带 His 标签的 mAFP 蛋白，并以此作为免疫原和筛选靶标，分别在 Balb/c 小鼠和 LOU 大鼠中筛选能特异性结合 mAFP 的单克隆抗体。本研究共制备 12 株稳定分泌小鼠抗 mAFP 和 8 株稳定分泌大鼠抗 mAFP 的单克隆抗体杂交瘤细胞株，通过间接化学发光法、原位蛋白杂交法和蛋白质免疫印迹法评估这些抗体与鼠源重组蛋白的反应性，同时采用间接免疫荧光法或蛋白质免疫印迹法分析这些抗体与稳定表达小鼠 AFP 的细胞株和小鼠肝癌细胞株 Hepa1-6 的反应性。研究结果发现，大鼠抗 mAFP 抗体中 6B8 和 10H3 能同时与线性和构象的 mAFP 蛋白反应，而小鼠抗 mAFP 抗体中仅 9G8 能与线性的 mAFP 蛋白反应。我们也建立了基于 CRISPR/Cas9 基因敲除技术的原发性肝癌小鼠模型，小鼠在尾静脉高压注射 p53/Pten 打靶质粒 4 个月后即可发展成为肝癌。在该小鼠模型中证实筛选获得的 6B8 和 10H3 这两株 mAFP 抗体可特异性识别小鼠血清和肿瘤组织中的 AFP 蛋白。

综上所述，本研究制备了可特异性识别 mAFP 的单克隆抗体，并在不同的细胞模型和原发性肝癌小鼠模型中评估 anti-mAFP 单克隆抗体的应用潜能，该研究为未来鼠肝癌模型的筛查、诊断及疗效评估提供有效检测手段，为以 AFP 为靶标的肿瘤疫苗设计奠定了基础。

关键词：肝癌；甲胎蛋白；单克隆抗体；免疫检测；鼠肝癌模型

Abstract

Liver cancer is one of the most common cancers, more than half new cases and deaths occur in China. According the data from National cancer registry in 2015, liver cancer is the fourth most common cancer with high incidence rate (466.1 per 100,000), and is the third leading cause of cancer death (422.1 per 100,000). Although great advances have been made in diagnosis and treatment of liver cancer during the last few years, liver cancer generally presents with poor prognosis and extremely low five-year survival rate. Most liver cancer patients are often diagnosed at advanced stage when distant metastases have presented, so the optimal opportunity for liver cancer treatment is missed. Besides, no effective treatment is available for most patients with advanced liver cancer. Therefore, it poses great significance for developing effective strategies to improve the efficacy of diagnosis and treatment

Alpha-fetoprotein (AFP) is a type of secreted glycoprotein, which normally expresses in fetal hepatocytes during the development of embryo and in liver cancer cells, but rarely expresses in mature hepatocytes. AFP is widely accepted as the tumor antigen for liver cancer, the AFP level in sera can be used in the screening, diagnosis, postoperative surveillance and follow-up study of liver cancer patients. Although the diagnostic sensitivity of AFP for liver cancer is about 60%, AFP is recognized as the most ideal biomarker for liver cancer so far. Recently, other biomarkers for liver cancer have been developed to further improve the diagnostic sensitivity of AFP. Moreover, epitope-optimized alpha-fetoprotein genetic vaccines have been proved to produce AFP specific memory $CD8^+$ T cell response. AFP has also been identified as a target for liver cancer immunotherapy.

Valuable pre-clinical assessment of oncolytic drugs that can be translated into clinical application requires autologous animal models with primary liver cancer, which are as relevant as possible to human pathology. Murine autologous liver cancer models established on immune-competent animals would mimic the patho-physiological

situation of HCC patients, can greatly facilitate the study of liver cancer. However, it usually takes at least 8 months to establish autologous animal models with primary liver cancer, thus it's urgent to establish a liver cancer model in a shorter period. Moreover, it is lack of detection reagents to screen, diagnose and evaluate the development of murine liver cancer in these models. The method for detecting mAFP would be a valuable tool for studying liver cancer.

The purpose of this study is to develop monoclonal antibodies (mAbs) against mAFP and preliminary evaluation of their potential application in the detection, diagnosis and treatment of liver cancer by using murine liver cancer cells and autologous liver cancer model. We employed Gibson Assembly systems to obtain recombinant plasmid, and then prepared recombinant mAFP antigen coupled with his-tag in vitro. Using the mAFP antigen as the immunogen, 12 hybridoma cell lines expressing mAbs against mAFP were obtained from Balb/c mice and 8 hybridoma cell lines expressing mAbs against mAFP were obtained from LOU rats.In addition, the binding activities of individual antibody were examined by the reaction with recombinant mAFP antigens using indirect CLEIA, dot blot and western blot assays, or the reaction with mAFP expressing cell lines or Hepa1-6 cells using indirect IFA and western blot assays respectively. It was found that 6B8 and 10H3 derived from rats can recognize both denatured and naïve mAFP, and 9G8 derived from mice can only recognize denatured mAFP. Lastly, we established a murine autologous liver cancer model within 4 months using CRIPSR/Cas9 system by inactivating the p53/Pten genes in the livers. We employed this model to evaluate the feasibility of these anti-mAFP mAbs in detecting mAFP in sera and tumors.

In summary, we obtained several monoclonal antibodies specifically against mAFP in both rats and mice, and evaluated the applicability of anti-mAFP mAbs on detecting the expression of mAFP in liver cancer cells and autologous liver cancer model. The results we obtained in this study would provide an effective detection tool for screening, diagnosis and efficacy evaluation for murine liver cancer and further facilitate the development of AFP based cancer vaccine.

Keywords: Liver cancer; Alpha-fetoprotein; Monoclonal antibodies; Immunological detection; Liver cancer model.

缩略表

缩写	英文全称	中文名称
AFP	alpha fetoprotein	甲胎蛋白
Amp	Ampicillin	氨苄青霉素
ATCC	American Type Culture Collection	美国模式菌种保藏中心
bp	Base Pair	碱基对
CLEIA	chemiluminescence immunoassay	化学发光酶免疫分析
DNA	Deoxyribonucleic acid	脱氧核糖核酸
DMSO	Dimethyl Sulfoxide	二甲基亚砜
FBS	Fetal Bovine Serum	胎牛血清
FITC	Fluorescein Isothiocyanate	异硫氰酸荧光素
GAM	Goat Anti-Mouse	山羊抗小鼠
GAR	Goat Anti-Rat	山羊抗大鼠
hAFP	Human alpha fetoprotein	人甲胎蛋白
HE	hematoxylin-eosin	苏木精-伊红
HRP	Horseradish Peroxidase	辣根过氧化物酶
H&T	Hypoxanthine & Thymidine	次黄嘌呤和胸腺嘧啶核苷
IF	Immunofluorescence	免疫荧光
IgG(M)	Immunoglobulin G(M)	IgG 抗体（IgM 抗体）
IHC	Immunohistochemistry	免疫组织化学
KDa	Kilo Daltons	千道尔顿
mAb	Monoclonal Antibody	单克隆抗体
mAFP	mouse alpha fetoprotein	小鼠甲胎蛋白
NBS	Newborn bovine serum	新生牛血清
NCBI	National Center for Biotechnology Information	美国国家生物技术信息中心
PBS	Phosphate Buffered Saline	磷酸盐缓冲液

PCR	Polymerase Chain Reaction	聚合酶链式反应
PEG	Polyethylene Glycol	聚乙二醇
RNA	Ribonucleic acid	核糖核酸
RPM	Revolutions per minute	转/分钟
WB	Western blot	蛋白质免疫印迹法
WHO	World Health Organisation	世界卫生组织